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ARTICLE

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Capacitively coupled electric fields accelerate proliferation of osteoblast-like primary cells and increase bone extracellular matrix formation in vitro

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Abstract Over the last few years, electric and electromagnetic fields have gained more and more significance in the therapy of bone fracture healing and bone disease. Yet, the underlying mechanisms on a cellular and molecular level are not completely understood. In the present study we have investigated the effects of capacitively coupled, pulsed electric fields on cellular proliferation, alkaline phosphatase activity, and matrix protein synthesis of osteoblast-like primary cells in vitro. Cells were derived from bovine periosteum and electrically stimulated by saw-tooth pulses of 100 V external voltage and 16 Hz frequency. This corresponds to an electric field of 6 kV/m across the cell membranes as could be shown by computer simulation. Field application caused acceleration of cell culture development. A significant increase of proliferation concurrent with an enhancement of alkaline phosphatase activity was observed in sub-confluent cultures. Exposure of confluent osteoblast-like primary cells to electric fields resulted in enhanced synthesis and secretion of extracellular matrix-related proteins. These findings suggest that capacitively coupled electric fields accelerate bone cell proliferation and differentiation in vitro and enhance the synthesis of cells leading to promoted matrix formation and maturation.

Key words Electrical stimulation · Osteoblast · Extracellular matrix · Collagen · Alkaline phosphatase

Introduction

Since Fukada and Yasuda reported the occurrence of piezoelectric potentials in mechanically loaded dry bone in the late 1960s (Fukada and Yasuda 1957), the idea of influencing bone growth and fracture healing by exogene electrical stimulation became challenging to orthopedists and scientists. Indeed, it could clearly be demonstrated in clinical and animal studies that osteogenesis can be induced and increased electrically in vivo (Akai et al. 1997; Alexa et al. 1996; Fourier and Bowerbank 1997; Grace et al. 1998; Huang 1997; Landry et al. 1997; Tabrah et al. 1998; Yonemori et al. 1996). Although the many early experiments in this field of research have led to the worldwide employment of electrical appliances in the therapy of bone disease, the mechanism of electric field-stimulated bone formation is not completely understood. In order to elucidate the underlying mechanisms of the field interaction on a cellular and molecular level a multitude of in vitro studies have been performed over the last 30 years. These studies were carried out using one of the following methods: (1) application of direct electrical currents (Wang et al. 1998), (2) capacitive coupling of electric fields (Binderman et al. 1985; Korenstein et al. 1984; Laub and Korenstein 1984; Ozawa et al. 1989; Rubin et al. 1996; Wang et al. 1998; Zhuang et al. 1997), and (3) inductive coupling of electromagnetic fields (Fitzsimmons et al. 1994, 1995a, b; Heermeier et al. 1998; Liu et al. 1997; Nagai et al. 1994). In in vitro studies many investigators reported on osteoblast-like primary cultures, giving a detailed description of cell isolation, cell proliferation and differentiation, and of initialization of mineral formation (Aronow et al. 1990; Auf'mkolk et al. 1985; Franceschi et al. 1994; Herbert et al. 1997; Masquelier et al. 1990; Rattner et al. 1997; Tenenbaum and Heersche 1982). It was found that untreated bone cells pass through a strict course of development. Owen et al. (1990) presented an extensive model of the relationship between proliferation and differentiation during the developmental sequence of rat osteoblasts in vitro. According to this model, bone cell development proceeds in three consecutive periods: (1) cell proliferation, (2) matrix development and maturation, and (3) mineral formation. The starting point and duration of each of these periods depend on the

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culture system and have to be determined experimentally.

The present study characterizes the effects of capacitively coupled electric fields on bone cells in vitro, determining the relevant electric field parameters at the cells' site by computer simulation. It is to find out whether the electric field is effective in a specific phase of cell development, e.g. during proliferation or differentiation of the cells, or if it has an effect on the whole course of osteoblast development. Beyond this, it is unclear how protein synthesis and formation of the extracellular matrix behave under the influence of electric fields. By simultaneously analyzing the cell reaction and the local field strength in a well-defined cell culture system using an appropriate arrangement for electrical stimulation these questions were systematically investigated.

Materials and methods

Cell culture

For culture initiation, we used the potential of osteoblasts to migrate from periosteum explants (Jones and Boyde 1977). The periosteal layer of calf metacarpus was aseptically stripped off the bone, cut into small pieces and plated into polystyrene culture dishes (13.5 cm in diameter) with its osteogenic side facing the bottom. High Growth Enhancement Medium (ICN Biomedicals GmbH, Eschwege, Germany) supplemented with 10% fetal calf serum, 250 µg/ml amphotericin B, 10,000 IU/ml penicillin, 10,000 µg/ml streptomycin and 200 mM L-glutamine (Biochrom KG seromed, Berlin, Germany) was used for culture maintenance and replaced once per week. The cultures were incubated at 37 °C in a humidified atmosphere of 95% air and 5% CO_2.

For electric field stimulation experiments, bovine primary cells were harvested by collagenase incubation (Biochrom KG seromed), counted using a coulter counter system (CASY I Model TT, Schärfe System GmbH, Reutlingen, Germany), and then transferred into new culture dishes at given inoculation densities, expressed as cell number (N) per ml of medium (cells/ml or N/ml). "Day of transfer" is referred to as day "0". Plating cells at low densities (< 300,000 cells/ml) led to sub-confluent spreading (< 40,000 cells/cm^2) resulting in cultures at their proliferation stage whereas osteoblasts being inoculated at higher concentrations (≈440,000 cells/ml) underwent confluent spreading (≈60,000 cells/cm^2) forming densely packed monolayers (see illustration in Fig. 1). After a four-day settling time allowing the cells to reattach to the bottom of the culture dish and after supplementing the original medium with 10 mM β-glycerophosphate (Sigma-Aldrich, Deisenhofen, Germany), the electric field stimulation was started.

In sub-confluent growth conditions, electrical treatment continued for 1 week. The cells then were harvested, counted with the coulter counter, and analyzed for ALP activity (see procedure below). Confluent cultures, however, were treated over different periods of time, i.e. for 5, 7, 9, 11, 13, and 18 days. Cells were analyzed with regard to their phenotype and to their synthesis activity by immunohistochemical staining (see procedure below). The experimental sequence is depicted in Fig. 1. Culture conditions and cell proliferation were routinely checked by light microscopy (Diaphot-TMD, Nikon Kogaku K. K., Tokyo, Japan).

Alkaline phosphatase determination

Quantification of ALP was performed by photometry of cell suspensions, utilizing the Alkaline Phosphatase Kit 104-LS by Sigma-Aldrich. Absorption was read at 400 nm using a spectral photometer by Beckman (Munich, Germany) and ALP activity was quantified by comparison with a calibration curve that was recorded simultaneously.

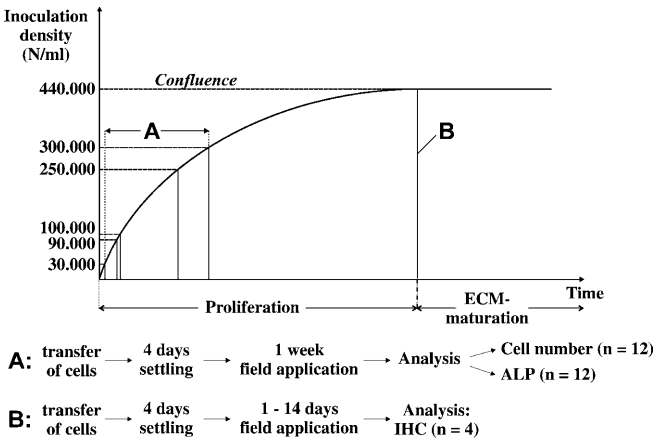

Fig. 1 Schematic illustration of the correlation between inoculation density and stage of cell culture development. Plating cells at different concentrations (plotted as ordinate) results in osteoblast cultures in different stages of development, expressed on a time-scale (x-axis). For studies at the proliferation stage, cells were inoculated into culture dishes at 30,000 cells/ml to 300,000 cells/ml leading to sub-confluent spreading (region A). To investigate during the matrix maturation period osteoblasts were plated confluently (440,000 cells/ml ≈60,000 cells/cm^2) (region B). The respective experimental procedures and the number n of culture dishes analyzed are depicted in the figure

Immunohistochemical staining

The cell monolayer was washed twice with PBS, fixed for 10 min with 100% methanol at –20 °C and left to air dry. ECM synthesis was assessed by immunohistochemical staining of ECM-related proteins, using monoclonal anti-osteocalcin, anti-osteonectin (Takara Biomedical Europe S.A., Gennevilliers, France), anti-proteoglycan (Chemicon Int. Inc. Temecula, Calif., USA), and polyclonal anti-bone sialoprotein (ImmunDiagnostik GmbH, Bensheim, Germany), anti-collagen type I (BioTrend Chemikalien GmbH, Köln, Germany), and anti-fibronectin (Collaborative Biomedical Products, Becton Dickinson Labware, Bedford, Mass., USA) antibodies. All antigens were simultaneously tested in each culture dish. In order to separate the staining areas from one another and to prevent merging of antibody solutions a grid was drawn upon the dried and fixed monolayer by means of a wax pen (PAP-Pen, DAKO Diagnostik GmbH, Hamburg, Germany). Thus, each of the above-mentioned ECM proteins was detected by its specific antibody in a separate frame (all of which had the same size). AEC (3-amino-9-ethylcarbazole) was employed as substrate-chromogen for light microscopy, resulting in a red-colored precipitate at the antigen site. The stained culture dishes were scanned and the digitalized image was loaded in an image processing program (Easy Win 32, Herolab, Wiesloch, Germany). Staining intensity was evaluated for each grid window separately and scaled by an internal intensity scale.

Electric field exposure and computer simulation

The experimental set-up shown in Fig. 2a is composed of a model HM8131-2 arbitrary function generator (HAMEG GmbH, Frankfurt am Main, Germany), a high voltage amplifier (Model 6827 A, Hewlett Packard), an oscilloscope (V-212, Hitachi), and 12

electrical capacitors, each consisting of a pair of circular (13 cm in diameter) high-grade steel electrodes mounted in a casing of insulating material. Culture dishes were placed on the lower electrode, being isolated from the medium by the polystyrene bottom of the dish. The upper electrode was positioned above the medium, leaving an air gap of approximately 2 mm (Fig. 2b). These set-ups were arranged in a CO_2-incubator allowing long-time field application on 12 culture dishes at the same time.

We applied an asymmetric saw-tooth voltage of 62.5 ms width with a repetition rate of 16 Hz. The signal was generated by the function generator and subsequently amplified, resulting in a peak-to-peak voltage amplitude of 100 V_{pp} at the capacitors.

In order to characterize the electric field across the cell monolayer, high speed SPICE computer simulations were carried out using the version ICAP/4Windows of Intusoft (San Pedro, Calif., USA). Applying 100 V_{pp} saw-tooth voltages as input signals at the capacitors, computer simulation showed a succession of identical voltage peaks of 100 μV across the cell monolayer (Fig. 3). Since the electrical resistivity of the cytoplasm is negligible compared with the resistivity of the cell membrane ($\rho_{membrane} \approx 10^7 \times \rho_{cytoplasm} \approx 1.3 \times 10^7$ Ω m (Glaser 1996) the 100 V voltage applied at the plate capacitor will mainly drop across the two (upper and lower) 8 nm lipid layers of the cell perpendicularly oriented to the external electric field. Thus, a 100 μV voltage corresponds to an electric field of 100 μV: (2× nm)≈6 kV/m across the cell membranes.

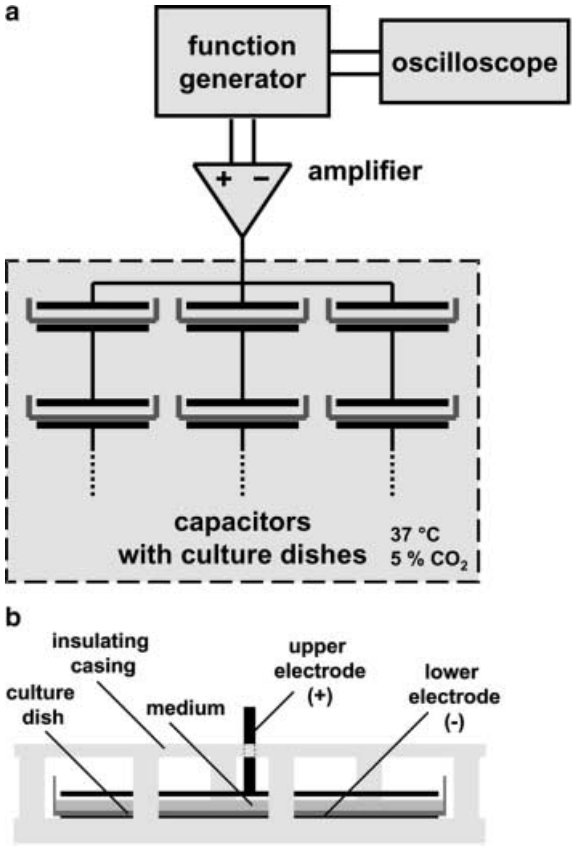

Fig. 2a, b Experimental set-up for electric field application. **a** The input signal is generated by the function generator and simultaneously visualized on the oscilloscope. A saw-tooth voltage of 5 V_{pp} is amplified by a voltage amplifier resulting in a 100 V_{pp} signal at the parallelly connected capacitors. **b** The culture dishes containing the adherent cell monolayers were placed between the pairs of electrodes constituting the capacitors. The whole set-up was stored in a CO_2-incubator

Statistical analysis

Values are means ± standard error of the mean and were compared by using Students' *t*-test.

Results

Characterization of bovine osteoblast-like primary cultures

Three to five days after culture initiation, precursor type cells started to migrate from the periosteal explants, forming a cell monolayer radiating onto the polystyrene surface of the culture dish. Four weeks after culture initiation, active growth slowed down and cells reached confluence at a density of approximately 60,000 cells/cm^2. Cessation of proliferation was followed by a rapid rise of ALP levels as can be seen in Fig. 4. In addition, confluent and post-confluent cultures showed high synthesis of collagen type I, bone sialoprotein, osteocalcin, and osteonectin as was ascertained by immunohistochemical staining of the cell monolayer.

Effects of electrical stimulation on proliferation and differentiation

In a first series of experiments, sub-confluently plated osteoblast-like primary cells were subjected to a one-

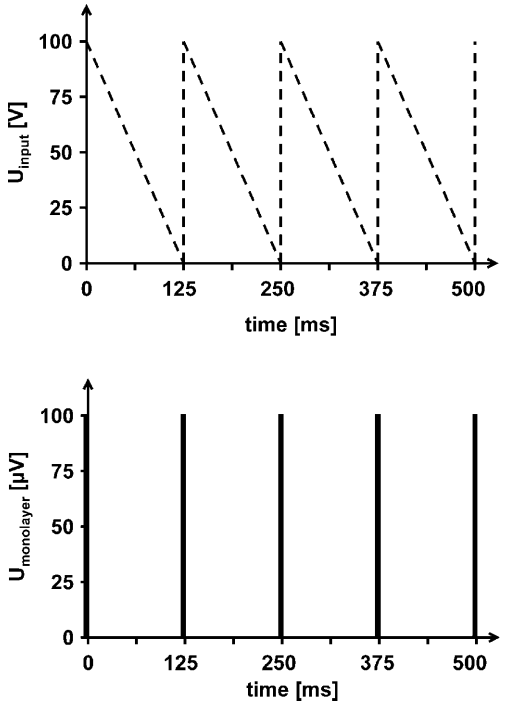

Fig. 3 Results of computer simulation based upon modeling the experimental set-up depicted in Fig. 2. Application of an external saw-tooth signal U_0 of 100 V_{pp} at the capacitors (U_{input}, *upper panel*) with a repetition rate of 16 Hz (period 62.5 ms) leads to a succession of 100 μV voltage peaks across the cell monolayer ($U_{monolayer}$, *lower panel*)

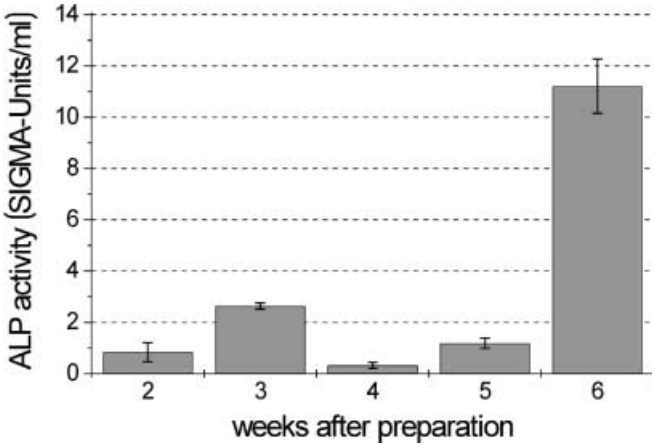

Fig. 4 ALP activity in bovine primary cell cultures ($n = 5$). Cells reached confluence four weeks after culture initiation at a density of approximately 60,000 cells/cm^2. Cessation of proliferation is followed by a rapid rise of ALP activity

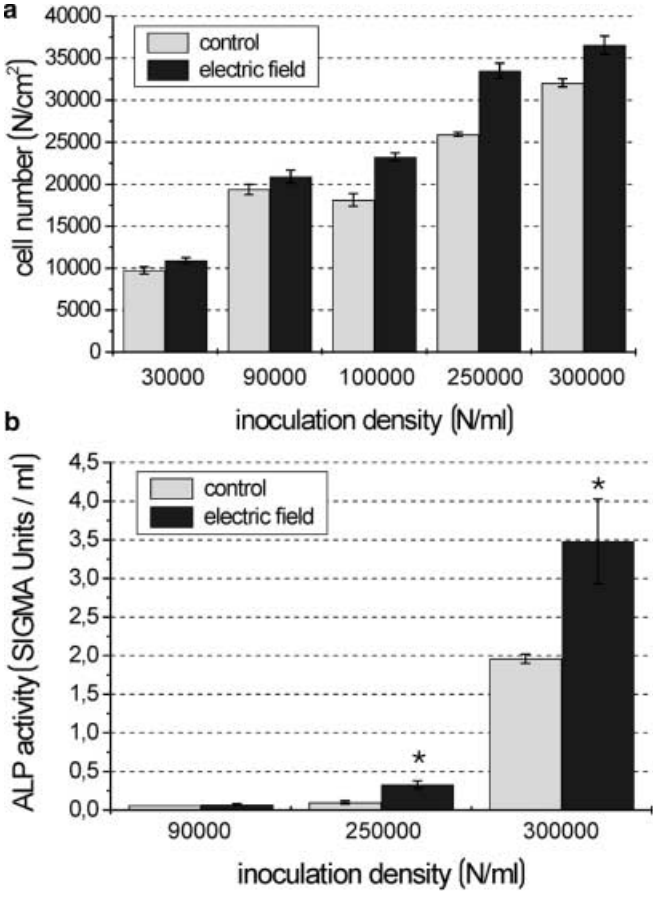

Fig. 5 a Effect of electric field stimulation on cell numbers in proliferating cultures. Bovine primary cells were transferred into culture dishes at specified inoculation densities and were exposed to continuous electric fields or to sham exposure conditions (controls) for a total of 7 days. 12 culture dishes were treated in each case ($n = 12$). After the one week period, cell numbers in electrically stimulated cultures were markedly elevated compared with non-stimulated cultures. This difference in cell numbers is statistically significant over the whole range from 30,000 cells/ml to 300,000 cells/ml ($P < 0.05$) and is maximal at medium inoculation densities of 100,000 cells/ml and 250,000 cells/ml ($P < 0.001$). **b** Effect of electric field stimulation on ALP activity in sub-confluent osteoblast-like primary cultures ($n = 12$). Continuous stimulation of bone cells by electric fields for one week results in a two- to three-fold higher enzyme activity at medium inoculation densities of 250,000 cells/ml ($P < 0.001$) and 300,000 cells/ml ($P < 0.05$). At low inoculation densities no statistically relevant difference can be observed (statistically significant differences of ALP activity are indicated by the symbol *)

week electrical treatment. Electrically stimulated cultures and (under all the same conditions) non-stimulated control cultures were analyzed for cell number and ALP activity. The results depicted in Fig. 5a show that the cell numbers in electrically treated cultures are significantly higher than those in the controls. At low inoculation densities (30,000 cells/ml and 90,000 cells/ml) and at very high densities (300,000 cells/ml) cell numbers of stimulated cultures exceed cell numbers of non-stimulated cultures by approximately 10% (significance level $P < 0.05$) whereas at medium inoculation densities (100,000 cells/ml and 250,000 cells/ml) cell numbers are even 30% higher in exposed than in unexposed cultures ($P < 0.001$). Figure 5b shows that osteoblast-like cells which had been treated by electric fields for one week exhibit higher ALP activity than untreated cells. At medium inoculation densities (250,000 cells/ml and 300,000 cells/ml), stimulated cells showed a two- to three-fold higher ALP activity than non-stimulated cells ($P < 0.001$ and $P < 0.05$, respectively). No significant difference in cell activity showed up in less proliferated cultures (90,000 cells/ml). ALP activity in cultures initiated at concentrations of less than 30,000 cells/ml were not detectable.

Effects of electrical stimulation on formation and maturation of the ECM

In order to investigate the effect of electric fields on the synthesis of extracellular matrix-related proteins, osteoblast-like primary cells were transferred into culture dishes at a cell density of approximately 60,000 cells/cm^2, representing the confluent state. Electrical stimulation was started four days after plating. For ECM characterization, electrically stimulated and non-stimulated cultures were analyzed immunohistochemically for several matrix proteins after different periods of treatment (1–14 days). In Fig. 6 protein synthesis is plotted as function of time for each matrix component.

As was shown in Fig. 5a cell number is significantly affected by electrical stimulation. Thus, this difference in cell number has to be taken into account when comparing the results of immunohistochemical staining of electrically treated and untreated cultures. We therefore determined the ratio of cell numbers in stimulated and control cultures ($N_{\text{control}}/N_{\text{electric field}}$) at all times and multiplied the values of staining intensity of the stimu-

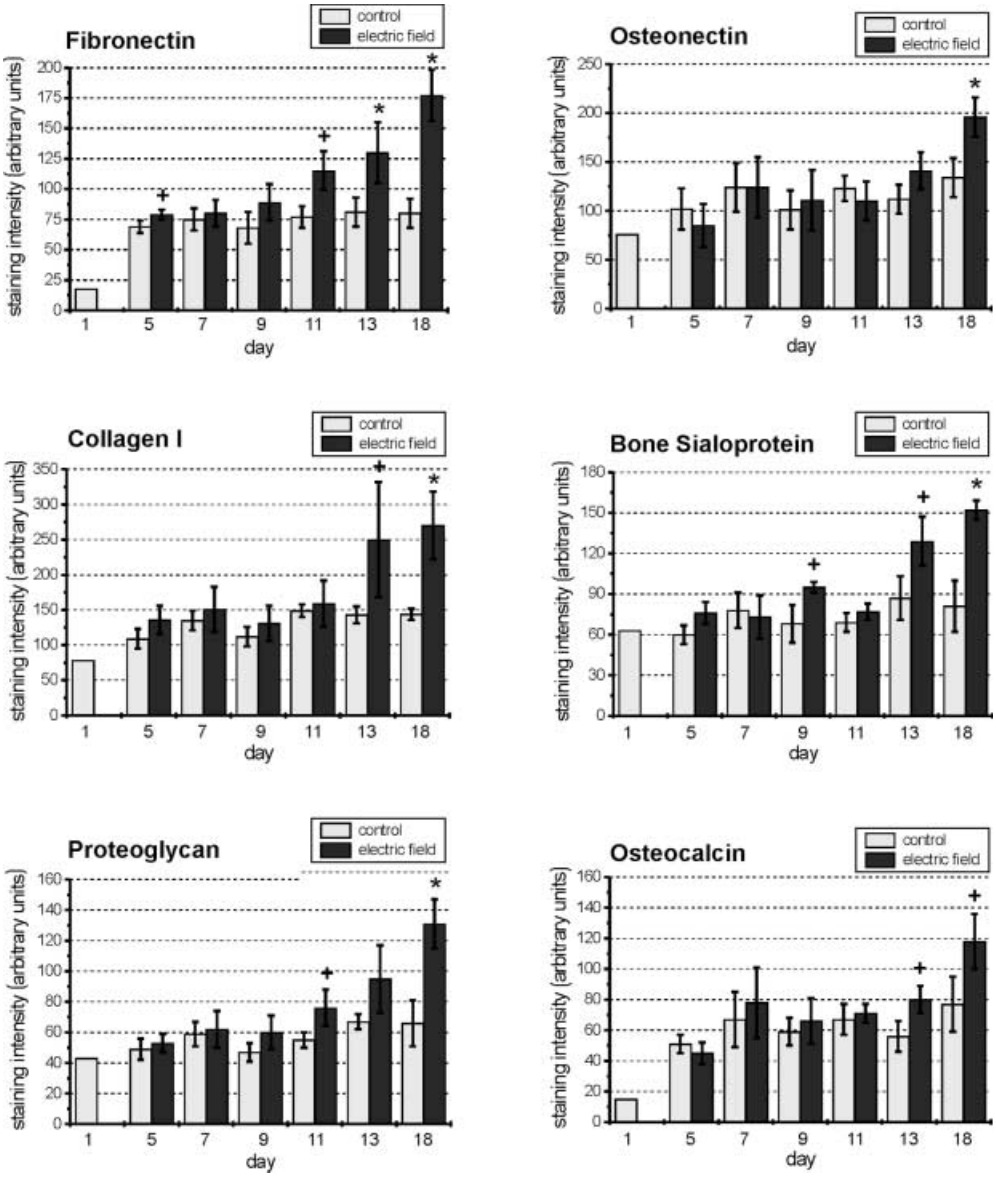

Fig. 6 Results of immunohistochemical staining of different ECM-related proteins. Osteoblast-like primary cells were plated confluently at day 0 and exposed to electric fields or to sham exposure conditions after a four-day settling time (day 4). After 5, 7, 9, 11, 13, and 18 days of treatment, cultures were immunohistochemically analyzed for fibronectin, osteonectin, type I collagen, bone sialoproteins, proteoglycans, and osteocalcin. Four culture dishes were handled at each time ($n = 4$). Staining intensities at the antigen sites were quantified by evaluating the digitalized images of the scanned culture dishes. Using an image processing program, stained areas were evaluated by an internal calibration scale of the program: higher scale values indicate stronger staining intensities. Staining intensities were standardized to the same cell number. A control culture taken one day after cell inoculation (day 1) served as reference. Application of electric fields leads to an increase of ECM-related protein synthesis, which is higher when cells are stimulated for longer times. Statistically significant differences of staining intensities between electrically treated and untreated cultures are indicated by the symbol $^{+}$ ($P < 0.1$) or * ($P < 0.05$)

lated cultures by this factor. In doing so, the values of staining intensity were standardized to the same cell number (that is the cell number of the control culture) allowing direct comparison of values obtained from stimulated and non-stimulated samples as shown in Fig. 6.

Comparison of the individual graphs shows that the expression of proteins is qualitatively similar for all markers investigated. In control cultures, ECM protein synthesis increased slightly from day "5" to day "18". Electric field application clearly enhances synthesis activity. Except for the first days, synthesis of each matrix protein is increased, reaching its largest values at the end of the two-week treatment.

Discussion

Characterization of bovine osteoblast-like primary cells

We adapted the method of Jones and Boyde (1977) for isolating osteoblast-like cells from bovine periosteum.

Cells derived from the periosteum explants showed distinct properties of the osteoblast phenotype as was confirmed by biochemical and immunohistochemical analysis. They exhibited uniform morphology, revealed distinct ALP activity and expressed high levels of bone sialoprotein, osteonectin, and osteocalcin, all of which are characteristic markers for the osteoblast phenotype (Aronow et al. 1990; Herbert et al. 1997). We observed a sequential development of the bovine primary cells being reflected in a temporal expression of these characteristic osteoblast parameters: as cell proliferation was down-regulated alkaline phosphatase activity increased more than five-fold about 6 weeks after culture initiation (see Fig. 4) and extracellular bone matrix proteins such as type I collagen, bone sialoprotein, osteocalcin, and osteonectin were synthesized in confluent (60,000 cells/cm^2) and post-confluent cultures (see Fig. 6, controls). Moreover, cells proved able to initiate mineralization in an advanced state of development, which is another key feature of the osteoblast phenotype (Tenenbaum and Heersche 1982, 1986). Thus, as was stated by Owen et al. (1990), osteoblasts pass through three distinct periods in vitro: (1) cell proliferation, (2) matrix development and maturation, and (3) mineralization.

Having been assured that bone cells derived from bovine periosteum develop from early proliferating pre-osteoblast-like cells into mature osteoblasts forming a collagenous matrix, the question arose whether it is possible to influence this developmental sequence by electric field stimulation. In this study, we concentrated on the proliferative and matrix maturation period. Cell number and ALP activity were chosen for characterizing the course of proliferation, and fibronectin, osteonectin, type I collagen, proteoglycans, bone sialoproteins, and osteocalcin served as markers for the period of matrix development and matrix maturation.

Effects of electrical stimulation

Capacitive coupling of electric fields is a suitable method for cell stimulation since it can be applied non-invasively in therapeutic applications. Regarding our experiments, capacitive coupling allows the calculation of field strengths and voltages at the cells' site. Since the electrodes are electrically insulated from the culture medium and the tissue, respectively, no electrochemical processes such as Joule heating (Jorgensen 1972), polarization (Bassett et al. 1964; Zengo et al. 1976), electrolysis and electroosmosis (Brighton et al. 1975) as well as infections (Steckel et al. 1984) can be induced as they were observed otherwise using direct electric currents. A disadvantage of field application by means of plate capacitors is the high voltage drops across the insulating layers so that only weak electric fields are active at the cells' site. In this paper, a voltage drop of 100 μV corresponding to an electric field strength of 6 kV/m was generated across the cells which proved to be sufficient to influence the cellular processes substantially.

The cell number in sub-confluent cultures having been exposed to electric fields for one week was markedly enhanced compared with the controls (Fig. 5a). This was maximal at medium inoculation densities (100,000–250,000 cells/ml) in which growth rate is high (Ozawa et al. 1989) suggesting that rapidly proliferating cells are particularly sensitive to electric field stimulation. A similar effect was observed in ALP activity showing that electric fields are able to increase significantly the enzymatic activity of the cells (see Fig. 5b). Stimulation reached its maximum in the middle of the period of proliferation. Since cultures initiated at low concentration (90,000 cells/ml $\approx$ 12,000 cells/cm^2) were not susceptible to the electric field whereas cells plated at $>250{,}000$ cells/ml ($>34{,}000$ cells/cm^2) showed a marked increase of ALP activity, it may be inferred that the primary osteoblasts have to overcome a lower threshold of cell density before there is a response to external electric fields. McLeod et al. (1993) proposed a limiting value of 30,000 cells/cm^2 which is in good agreement with the observations presented in this study.

The reason for the dependence of cell number and ALP stimulation on cell density may be a physical one. During early proliferation, cells are only sparsely scattered over the bottom of the culture dish, leaving some areas of the bottom in direct contact with the culture medium. In these regions, the currents induced by the saw-tooth pulses will be shunted by the highly conductive medium, by-passing the insulating single osteoblasts. As proliferation proceeds a compact layer of high electrical resistance will be formed. Since collagen production is proportional to ALP activity (Farley et al. 1991) the electrically induced increase of enzyme activity implies stimulation of collagenous matrix synthesis and deposition. This simultaneous growth of the organic extracellular matrix further enhances the efficiency of the electric field.

As we observed in densely packed osteoblast-like cells plated at 60,000 cells/cm^2 the electric field also had a stimulating effect on the subsequent period of matrix maturation. Immunohistochemical staining of several relevant extracellular marker proteins revealed the synthesis behavior depicted in Fig. 6. While cell activity in the controls rose only slightly over the two-week treatment (day 5–18) electrical stimulation of confluent cultures resulted in an enhancement of ECM-related protein synthesis. Osteonectin and fibronectin are prominent attachment proteins responsible for cell adhesion (Lane and Sage 1994; Winnard et al. 1995) while type I collagen, proteoglycans, and bone sialoproteins are considered as important structural proteins contributing to the formation of the ECM (Owen et al. 1990; Robey 1996), and osteocalcin, a marker for the more differentiated cell, indicates the transition to mineral formation (Owen et al. 1990). Thus, electrically induced elevation of fibronectin and osteonectin reveals increased cell adhesion and cell–matrix interaction, elevation of type I collagen, proteoglycans, and bone sialoproteins indicates enhanced matrix formation and modification, and the rise in osteocalcin points to the

progressive maturation of the osteoblast-like cells The matrix protein production in electrically stimulated cultures further increased with longer lasting field application but not before the cells had grown for approximately one week. This induction period may be attributed to the same factors as we discussed on the field effect on proliferating cells: electric coupling can obviously not take place until the osteoblasts have attached to the polystyrene bottom and have formed a compact cell monolayer of high electrical resistance.

In summary, this report demonstrates that application of a 100 V_{pp} external voltage signal by means of an outer plate capacitor induces an electric field of 6 kV/m across the cell monolayer which significantly enhances proliferation, maturation, and ECM protein synthesis of osteoblasts in vitro. This paper is the basis for further studies following the elucidation of the primary field interaction at the cell membrane. Application of a well-defined, local electric field and simultaneous observation of the cell reaction, e.g. in a single-cell system in which membrane properties can be easily studied, should allow the well-aimed investigation of the underlying coupling mechanisms and the resulting changes of gene activation. Assuming an electromechanical transduction mechanism based on the piezoelectric behavior of bone (Yasuda 1953) it remains to be determined whether the stimulation is triggered (1) primarily by mechanoreceptors detecting the electrically induced deformation of the cell or (2) secondarily by specific membrane receptors measuring the mechanically induced piezoelectric and streaming potentials. In order to investigate this, electrical stimulation experiments will be complemented by mechanical stimulation experiments.

Only if both the field-induced processes and the electric field strength at the cells' site are known, will it be possible to determine the field parameters that are required for a secure, controlled and optimized application of the stimulation technique in the therapy of bone fracture healing and bone disease.

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